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RADIOIMMUNOASSAY FOR QUANTITATIVE DETERMINATION OF MORPHINE IN CAPSULES OF *PAPAVER SOMNIFERUM*

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Key Word Index—*Papaver somniferum*; Papaveraceae; poppy alkaloids; extraction and quantitation; radioimmunoassay; morphine.

Abstract—A radioimmunoassay (RIA) procedure for the determination of pmol quantitites of morphine in capsule samples of *Papaver somniferum* was developed. An antiserum developed against a conjugate of morphine-3-hemisuccinate-BSA was relatively specific for morphine and possessed moderated cross-reactivity with codeine and mild cross-reactivity with thebaine, but none with narceine, papaverine, or noscapine. The standard curve was linear over a range of 0.01–0.20 ng. This assay allows for the rapid, sensitive and precise determination of morphine in unpurified aqueous extracts of capsule samples. The amounts of morphine in the aqueous extracts determined by radioimmunoassay were validated by high performance liquid chromatography (HPLC). The two methods show a high correlation coefficient ($r = 0.98$) with no significant difference in determinations of morphine content by RIA and HPLC.

INTRODUCTION

The biosynthesis of thebaine, codeine and morphine recently have been investigated in cell cultures of *Papaver somniferum* [1–3]. Efficient and sensitive methods are needed for quantitating these metabolites, since they accumulate in only small amounts. Radioimmunoassay (RIA) procedures have been applied in studying biotransformations and analgesic properties of narcotic drugs in man and animals [4, 5], but have not been used to detect the secondary metabolites of morphine alkaloids in plants. RIA provides a specific method for rapid quantitative determination of compounds of biological interest [6]. However, RIA does not provide accurate quantitation of a compound if the immunological behavior of interfering compounds is similar to that of the antigen.

In this study, we have investigated RIA as a means of determining the morphine content in extracts of alkaloids of capsule samples of *P. somniferum*. The morphine-3-hemisuccinate (M-3-HS) derivative was coupled to bovine serum albumin (BSA) and used as the antigen. This antigen was similar to the antigen described by Wainer *et al.* [7] except that we conjugated nearly twice as much morphine per molecule of BSA. Thus, the antibody we prepared appeared to be more specific than that of Wainer *et al.* [7]. We also validated RIA data with the results obtained from HPLC. Various extraction methods were investigated in order to optimize the extraction procedure for RIA.

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RESULTS AND DISCUSSION

Comparison of methods for extracting morphine from capsule samples

Ideally, a simple, rapid and reproducible method for extracting morphine from capsule samples is desirable as the first step of an analysis based on RIA. Table 1 shows the results of HPLC analyses of the 18 capsule samples extracted by two different methods. Although comparison by paired *t*-test showed that the amount of morphine content obtained from Vincent and Engelke's [8] extraction method was significantly ($P < 0.05$) less than the amount of morphine obtained from microextraction, this difference was smaller than the variation obtained from the replicate extractions by the same method. The method developed by Vincent and Engelke [8] extracts five major alkaloids, including narcotine, papaverine, thebaine, codeine and morphine, and also some other minor alkaloids. The major alkaloids in the extract can be determined by HPLC, but this extraction is rather time consuming. The microextraction technique described herein is a modification of Vincent and Engelke's method, and provides the capability of manipulating smaller samples of capsule material. Determinations of morphine by HPLC from microextracts (Table 1) compared well with the values obtained by Vincent and Engelke's [8] method. However, both methods extracted other alkaloids that interfered with the RIA procedure. As discussed later, an adaptation of Wu and Wittick's [9] aqueous extraction procedure was necessary for the preparation of extracts for RIA.

Antigen properties

Morphine was converted to morphine-3-hemisuccinate (M-3-HS) by reaction of the free base with succinic

Table 1. HPLC analysis of morphine content of extracts of capsules of *P. somniferum**

Sample No.	Morphine content (mg/g dry capsule): Method of Vincent and Engelke [8]	Microextraction procedure
1	0.60	0.70
2	2.80	3.04
3	2.60	2.80
4	1.50	1.45
5	1.80	1.60
6	2.34	2.34
7	0.80	0.81
8	1.45	1.51
9	1.00	1.21
10	2.34	2.81
11	2.36	2.60
12	1.10	1.15
13	1.40	1.62
14	1.90	2.69
15	1.40	1.58
16	1.80	1.87
17	3.22	3.33
18	2.48	2.32

*The correlation constant ($r = 0.96$) between these two methods was determined by the least squares method.

anhydride in pyridine (Fig. 1). The M-3-HS was positive to the Dragendorff test [10] and negative to the Pauly test [11]. The IR spectrum of the product contained bands at 1730 cm^{-1} (ester carbonyl) and at 1550–1660 cm^{-1} (carboxylate). The mass spectrum showed a M^+ (m/z) of 385. M-3-HS was conjugated to BSA by the mixed anhydride method [12] and the number of molecules of morphine conjugated per molecule of BSA was determined by hydrolysing M-3-HS–BSA under basic conditions. The free morphine that was generated was extracted by chloroform-*iso*-propanol (3:1) and the concentration of free morphine was determined by HPLC [8]. Based upon the morphine determined by HPLC, the average ratio of conjugation was 13.5 molecules of morphine per molecule of BSA (assuming a MW of 70 000). In our preparation, over twice as much morphine was conjugated to BSA than was reported for a similar preparation by Wainer *et al.* [7] who indicated that an average of 6.5 molecules of morphine, at most, were bound per molecule of BSA. For RIA, the anti-M-3-HS–BSA serum obtained (after 3 months' immunization) required a dilution of 1:500 (Fig. 2) to reach 40% of total binding compared to a dilution of 1:300 for the antigen (M-3-HS–BSA) [7]. Other antigens, 3-*O*-carboxymethylmorphine [13], or *N*-carboxypropylnormorphine [15] required 1:200 or 1:350 dilution, respectively, to reach the same degree of total binding (40%). The reason for this difference in antiserum titer may be due to the position and the degree of conjugation of morphine molecules to BSA.

The cross-reactivities of various alkaloids with M-3-HS–BSA antiserum is shown in Fig. 3. The various alkaloids used in this study are the major alkaloids that are contained in capsules of *P. somniferum*. The extent of

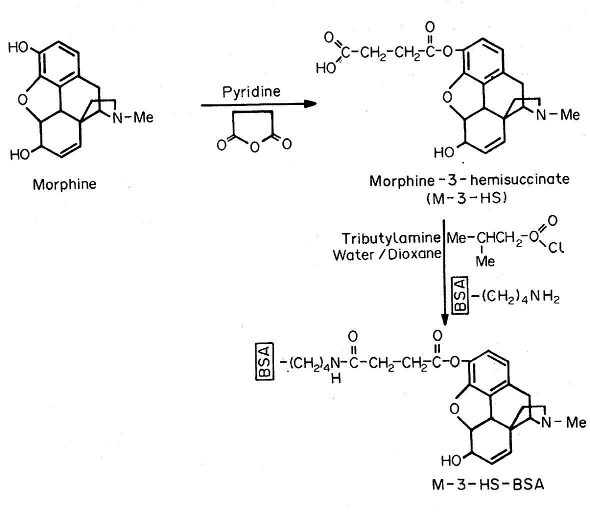

Fig. 1. Synthesis of immunogenic morphine-3-hemisuccinate–BSA conjugates.

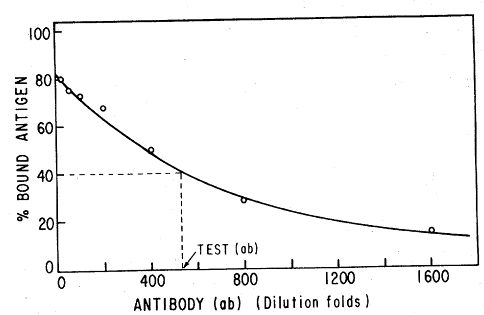

Fig. 2. Antibody dilution curve. Antiserum bled from the rabbit was diluted to various concentrations as indicated. RIA was then performed as described in the Experimental with these various dilutions of antiserum. The percentage of bound morphine was determined by comparison of the radioactivity from the incubation with antiserum.

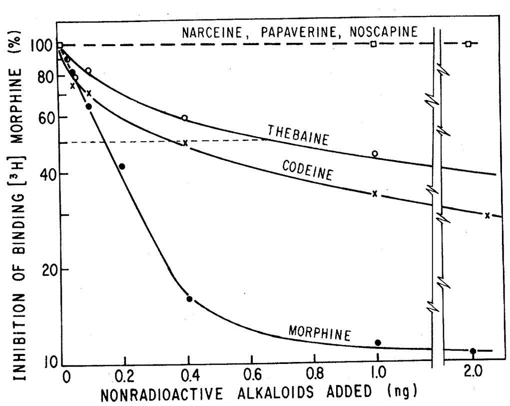

Fig. 3. Competition of [^{3}H]morphine by various alkaloids. Increasing concentrations of nonradioactive morphine (●——●), codeine (×——×), thebaine (○——○), narceine, papaverine and noscapine (□---□) were included in the standard RIA assay as described in the Experimental. The inhibition of binding of [^{3}H]morphine to antiserum was expressed as a percentage compared to that from incubation without nonradioactive alkaloid added.

binding of various opium alkaloids to antibody was determined by incubating the alkaloids with antibody in the presence of labeled morphine and measuring the inhibition of antibody–[^{3}H]morphine complex formation. Major alkaloids that are present in capsule extracts, such as morphine, codeine and thebaine, were all bound to antibody. The concentration required to cause a 50% inhibition of the antibody–labeled antigen complex varied with each alkaloid. In order to produce a 50% inhibition, an amount of codeine over twice as great as morphine (0.37 and 0.15 ng, respectively) and an amount of thebaine nearly five times (0.63 ng) greater than morphine were required. Other alkaloids, such as narceine, papaverine and noscapine, showed no inhibition of the [^{3}H]morphine–antibody binding.

Antibody generated from the 3-*O*-carboxymethyl morphine–BSA [13] had the same binding affinity for morphine and codeine, while antibody derived from *N*-carboxypropylnormorphine [15] showed a much lower affinity for codeine than morphine (a concentration of 400 times more codeine than morphine was required to reach the same degree of binding). Therefore, the specificity of each antibody seemingly depends on the position, number of morphine molecules conjugated to BSA and the distance between BSA and the morphine molecule.

Sensitivity of RIA

The sensitivity of RIA for determining morphine in the alkaloid extracts of *P. somniferum* is shown on Fig. 4. The assay is based on the principle that the antibody has a limited number of binding sites, therefore, both unlabeled and radioactive morphine will compete equally for the binding sites on the antibody. The addition of increasing amounts of unlabeled morphine to a fixed amount of [^{3}H]morphine and antibody resulted in competitive inhibition of the labeled morphine for the formation of antibody–hapten complex. The sensitivity of this application of RIA is revealed in Fig. 4. The addition of 0.15 ng unlabeled morphine caused a 50% displacement of the labeled morphine from the antibody. The variability of the assay was ±5% at all concentrations.

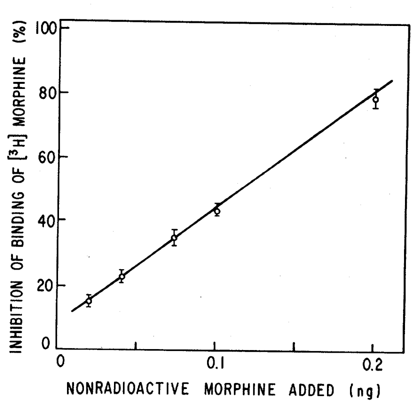

Fig. 4. Inhibition by nonradioactive morphine of the binding of [^{3}H]morphine by antiserum to a morphine-3-hemisuccinate–BSA conjugate. Various concentrations of nonradioactive morphine were included in the standard RIA as described in the Experimental. The data are mean values of triplicate assays; vertical bars indicate the s.d. which was less than 5%.

Comparison of RIA and HPLC determination of morphine content

Morphine obtained by the microextraction procedure from 12 samples was analysed both by HPLC and RIA. A paired *t*-test showed that HPLC results were significantly ($P < 0.01$) less than RIA, which indicated poor agreement between these two methods.

As mentioned previously, we found that antibodies in our preparation cross-react substantially with codeine, thebaine and possibly other unidentified alkaloids. In the presence of these interfering alkaloids, precise determination of morphine by RIA is not possible. To obtain an extract suitable for analysis by RIA, the original extraction procedures described by Wu and Wittick [9] were adapted for extracting morphine in an aqueous phase while leaving other alkaloids in the methylene chloride phase. We designed the procedure to determine morphine amounts of 0–0.2 ng. The variability of standards or samples analysed in triplicate averaged less than 5%. To demonstrate the reliability of the RIA procedure, 20 samples of the alkaloid, extracted by the modified Wu and Wittick's method, were analysed by RIA and by HPLC (Fig. 5). RIA determinations were performed on an aliquot of the aqueous extracts of capsule samples. Both methods correlated closely over the whole range of concentrations found in the samples ($r = 0.98$). Paired *t*-test results indicated that there was no significant difference between RIA and HPLC. Compared to HPLC, RIA requires minimum procedural effort and several hundred determinations may be made per day by an analyst.

From the results described above, RIA can be applied successfully for screening the morphine content of capsules of *P. somniferum*. The accuracy of determining morphine from the alkaloid extracts is very dependent on the nature of the antibody used. Various extraction methods could be utilized to eliminate the compounds that interfere with the determination of morphine by RIA, but the aqueous extraction described herein was entirely satisfactory. The RIA method described herein possesses the unique features of high sensitivity (usually < 10 ng) which is 1000-fold more sensitive than conventional HPLC or TLC. The capacity of the RIA procedure to accommodate the analysis of large numbers of samples of *P. somniferum* for morphine content, with minimum effort on the part of the analyst, represents a major improvement over current HPLC or TLC methods which are more time consuming. With the RIA procedure, the mass

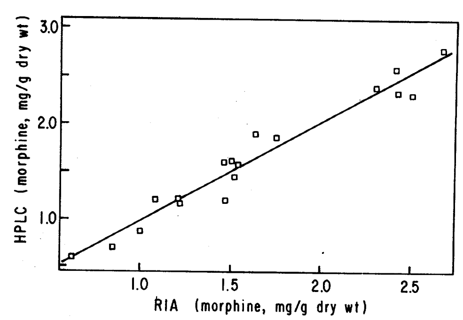

Fig. 5. Correlation between RIA and HPLC for the quantitative determination of morphine from capsule samples of *P. somniferum*.

screening of morphine content of *P. somniferum* in breeding programs becomes possible. The potential of RIA for morphine determination can be extended to the screening of cell cultures [14] and to kinetic studies of enzymes involved in morphine metabolism [15].

EXPERIMENTAL

Thebaine, codeine and morphine were gifts from Penick Corporation, NJ.* The alkaloids, pepaverine, narceine and noscapine were obtained from Applied Science Laboratories. Incomplete adjuvant was purchased from Difco Laboratories. BSA was obtained from Sigma Chemicals Inc. Radioactive $[1(N)\text{-}^3H]$morphine (S.A. 20–30 Ci/mmol) was obtained from Amersham and scintillation cocktail 'Riafluor' from New England Nuclear. All other chemicals were analytical grade and from commercial sources. HPLC and alkaloid extraction solvents were from commercial sources and were Mallinckrodt Nanograde or equivalent.

Microextraction of total alkaloids. The microextraction method was a modification of the method described in ref. [8]. To 100 mg of dried and pulverized sample was added 2 ml 5% HOAc. The suspension was agitated with a vortex mixer then sonicated and 200 μl conc. NH_4OH was added. The suspension was allowed to stand for 30 min, then extracted by shaking with 5 ml $CHCl_3$–*iso*-PrOH (3:1) for 20 min, the mixture was then centrifuged (*ca* 2500 rpm) for 3 min. The $CHCl_3$ layer was collected and the aq. phase extracted twice according to the above procedure. The $CHCl_3$ phases were combined and passed through a Pasteur pipette plugged with glass wool and packed with dry Na_2SO_4 to a depth of 1 cm. This extract was then evaporated to dryness under N_2 and the residue redissolved in 0.5 ml EtOH. A 25 μl aliquot of the EtOH soln was analysed by HPLC [8] with minor modification to determine the morphine content. A HPLC equipped with a stainless steel column (30 cm × 3.9 mm id) packed with 5 μm porous Si gel, a multiple wavelength UV–visible detector and a syringe-loading injector with 100 μl capacity was used. The solvent system was *n*-hexane–$CHCl_3$–EtOH–Et_2NH (60:6:8:0.1) with column temp. 25°, flow rate 2 ml/min and detector wavelength 285 nm.

Preparation of aqueous extract for morphine determination by RIA. The extraction procedure was a modification of the method described in ref. [9]. Powdered capsule material (100 mg) was allowed to soak overnight in 10 ml H_2O. The mixture was then dispersed by stirring and ultrasonic agitation and centrifuged at 10 000 rpm for 10 min. The centrifugate was thoroughly washed twice with 5 ml H_2O. After recentrifugation, the supernatants were combined and H_2O was added to make a final vol. of 25 ml. To 100 μl of this aq. extract was added 20 ml H_2O, 0.2 g $Ca(OH)_2$ and 10 ml of CH_2Cl_2 and the mixture centrifuged for 10 min at 5000 rpm. The aq. layer was removed and 10 drops of HOAc were added to 15 ml of this aq. phase which was then diluted to 25 ml with H_2O. Any turbidity occurring during the dilution was cleared by the addition of a few drops of HOAc. This dilute, aq. extract was used to determine morphine content by RIA.

Preparation of morphine-3-hemisuccinate–BSA conjugate. Morphine-3-hemisuccinate (M-3-HS) was synthesized by reacting morphine with succinic anhydride in pyridine [7]. Crystalline M-3-HS (mp 237–239° decomp.) was obtained and recrystallized twice from 60% EtOH. TLC in EtOAc–MeOH–NH_4OH (17:2:1) indicated that M-3-HS remained at the origin while morphine migrated with an R_f of 0.3. M-3-HS was conjugated to BSA by the mixed anhydride method [12] to yield the conjugate (M-3-HS–BSA).

Immunization method. Four New Zealand white female rabbits, weighing *cu* 2 kg each, were immunized by intramuscular injections at multiple loci of 2 mg M-3-HS–BSA in 0.9% saline emulsified with an equal vol. of incomplete adjuvant as antigen. Second injections with the same amount of antigen were made 1 week later. The rabbits were bled once per week for 10 weeks to collect sera for antibody titer assay. If additional antibody was needed, the same rabbits were immunized with the same amount of antigen and bled at weekly intervals. In general, a high antibody titer was obtained 2 weeks after the booster inoculation.

Characterization of antisera. Prior to running RIA, antisera were characterized as follows. (1) Antiserum titers were determined by incubation of 100 μl of various dilutions of antiserum in 0.1% gelatin–PBS buffer (0.05 M KH_2PO_4–0.15 M NaCl–0.03% sodium azide) with 100 μl $[^3H]$morphine (87 pg, *ca* 10 000 cpm) in 0.1% gelatin–PBS buffer and 100 μl 0.1% gelatin–PBS buffer. The mixture was incubated overnight at 0–4° and antibody bound radiotracer was separated from free tracer by the addition of 500 μl of an ice-cold, stirred suspension of activated charcoal (10 mg charcoal/ml 0.1% gelatin–PBS buffer). The tubes were centrifuged at 3500 rpm for 5 min at 0–4°. A 500 μl aliquot of the clear supernatant and 10 ml Riaflour liquid scintillation fluid were added and radioactivity was determined by liquid scintillation counting. The antiserum titer chosen for RIA was the dilution that bound 40% of the total radiotracer added. (2) For cross-reactivity studies, the above procedure was used except the 100 μl 0.1% gelatin–PBS buffer was replaced by 100 μl of increasing concns of appropriate nonradioactive alkaloids in the same buffer, and antiserum added was the titer (1:500, final dilution 1:1500) that contained antiserum sufficient to bind 40% of the total radiotracer added.

Standard RIA for morphine determination in alkaloid extracts. (a) Alkaloid extracts obtained from the microextraction. An aliquot of the alkaloid extracts obtained from the microextractions was diluted × 10^5 with EtOH, then 10 μl (or 20 μl) of the diluted sample was reduced to dryness under N_2 and the residue resuspended in 0.1% gelatin–PBS buffer (100 μl). RIA was performed by incubating the above alkaloid extracts in 0.1% gelatin–PBS buffer (100 μl), with antiserum (100 μl, 1:500 dilution) and radioactive antigen (10 000 cpm in the same buffer) at 0–4°. The bound and free antigens were separated as described above. Background and nonspecific binding were estimated by leaving out the 0.1 ml aliquot of rabbit antiserum which contained the specific antibodies. The cpm obtained from the sample were subtracted from the background. All RIA were performed in triplicate. (b) Alkaloid extracts obtained from the modified Wu and Wittick's method. To an aliquot (10 or 20 μl) of the aq. extract was added 0.1% gelatin–PBS buffer to make a final vol. of 100 μl. RIA was carried out as described above.

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*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

REFERENCES

1. Furuya, T., Ikuta, A. and Syono, K. (1972) *Phytochemistry* **11**, 3041.
2. Furuya, T., Nakano, M. and Yoshikawa, T. (1978) *Phytochemistry* **17**, 891.
3. Tam, W. H. J., Constabel, F. and Kurz, W. G. W. (1980) *Phytochemistry* **19**, 486.
4. Spector, S. (1971) *J. Pharmacol. Exp. Ther.* **178**, 253.

5. Findlay, J. W. A., Butz, R. F. and Welch, R. M. (1977) *Res. Commun. Chem. Pathol. Pharmacol.* **17**, 595.
6. Yallow, R. S. (1978) *Science* **200**, 1236.
7. Wainer, B. H., Fitch, F. W., Rothberg, R. M. and Fried, J. (1972) *Science* **176**, 1143.
8. Vincent, P. G. and Engelke, B. F. (1979) *J. Assoc. Off. Analyt. Chem.* **62**, 310.
9. Wu, C. Y. and Wittick, J. J. (1977) *Analyt. Chem.* **49**, 359.
10. Munier, R. (1953) *Bull. Soc. Chim. Biol.* **31**, 1225.
11. Stantavy, F. (1969) in *Thin Layer Chromatography* (Stahl, E., ed.) pp. 421–471. Springer, New York.
12. Vunakis, H. V., Wasserman, E. and Levine, L. (1972) *J. Pharmacol. Exp. Ther.* **180**, 514.
13. Spector, S. and Parker, C. W. (1970) *Science* 1347.
14. Weiler, E. W. (1977) in *Plant Tissue Culture and its Bio-Technological Application* (Barz, W., Reinhard, E. and Zenk. M. H., eds.) pp. 266–277. Springer, Berlin.
15. Treimer, J. F. and Zenk, M. H. (1978) *Phytochemistry* **17**, 227.